Effects of the Level of mRNA Expression on Biophysical Properties, Sensitivity to Neurotoxins, and Regulation of the Brain Delayed-Rectifier K^+ Channel Kv1.2†

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ABSTRACT: Injection of 0.2 ng of cRNA encoding the brain Kv1.2 channel into *Xenopus* oocytes leads to the expression of a very slowly inactivating K^+ current. Inactivation is absent in oocytes injected with 20 ng of cRNA although activation remains unchanged. Low cRNA concentrations generate a channel which is sensitive to dendrotoxin I (IC_{50} = 2 nM at 0.2 ng of cRNA/oocyte) and to less potent analogs of this toxin from *Dendroaspis polylepis* venom. A good correlation is found between blockade of the K^+ current and binding of the different toxins to rat brain membranes. High cRNA concentrations generate another form of the K^+ channel which is largely insensitive to dendrotoxin I (IC_{50} = 200 nM at 20 ng of cRNA per oocyte). At low cRNA concentrations, the expressed Kv1.2 channel is also blocked by other polypeptide toxins such as MCD peptide (IC_{50} = 20 nM), charybdotoxin (IC_{50} = 50 nM), and β-bungarotoxin (IC_{50} = 50 nM), which bind to distinct and allosterically related sites on the channel protein. The pharmacologically distinct type of K^+ channel expressed at high cRNA concentrations (20 ng of cRNA/oocyte) is nearly totally resistant to 100 nM MCD peptide and hardly altered by charybdotoxin and β-bungarotoxin at concentrations as high as 1 μM. Both at low and at high cRNA concentrations, the expressed Kv1.2 channel is blocked by an increase in intracellular Ca^{2+} from the inositol trisphosphate sensitive pools and by the phorbol ester PMA that activates protein kinase C.

Electrophysiological studies have identified a large number of voltage-sensitive K^+ channels which play an important role in shaping the electric activity of extiable cells. These K^+ channels differ in their kinetics, voltage dependences, and unitary conductances and in their pharmacology and particular functions (Rudy et al., 1991; Rehm, 1991).

The very large diversity of properties of voltage-sensitive K^+ channels in mammals is now known to be due (i) to the existence of at least 19 genes, (ii) to alternate splicing [for a review see Jan and Jan (1990)], and (iii) to the heterologous association of subunits encoded by different genes (Isacoff et al., 1990) to form the tetramers which are the basic functional structures of voltage-sensitive K^+ channels (MacKinnon, 1991; Rehm & Lazdunski, 1988a).

Another element of diversity for the voltage-dependent K^+ channels is the presence in their structure, in addition to the large (60–90 kDa) α–subunits (Betz, 1990), of a smaller ~38-kDa β-subunit (Rehm & Lazdunski, 1988a). α-Subunits are the only ones that have been sequenced up till now and are sufficient to form a functional K^+ channel. However, β-subunits could modify the basic functional properties of α-subunits as previously observed for small subunits of voltage-dependent Ca^{2+} and Na^+ channels (Pragnell et al., 1991; Isom et al., 1992; Miller, 1992; Hullin et al., 1992).

A newly discovered and apparently very important element of the K^+ channel activity is associated with the level of expression of the corresponding messenger RNA. Oocyte expression of the cRNA for the major K^+ channel in T-lymphocytes (Kv1.3; Attali et al., 1992) has been shown to produce either a transient or a sustained K^+ current, which is either charybdotoxin- (ChTx-)[1] sensitive or ChTx-resistant at low or high levels of cRNA, respectively (Honoré et al., 1992). Similarly, a K^+ channel cDNA of the *Shaker* subfamily (ShH4) has been shown to express in *Xenopus* oocytes not only a transient A-type K^+ current but also, at an increased level of expression, a noninactivating K^+ current with a markedly reduced sensitivity to tetraethylammonium (Moran et al., 1992).

One of the purposes of this work is to check whether the modulation via the level of mRNA expression of the biophysical and pharmacological properties of K^+ channels is also observed with the K^+ channel Kv1.2. This voltage-sensitive K^+ channel has been chosen for this work for several reasons. (i) This channel has a rich pharmacology and a high sensitivity to three different types of polypeptide toxins—dendrotoxin I (DTX_I) from snake venom, mast cell degranulating (MCD) peptide from bee venom, and ChTx from scorpion venom—which are known to bind to the same protein subunit at three distinct but allosterically related sites (Bidard et al., 1989; Schweitz et al., 1989a; Stühmer et al., 1989) and which, by inhibiting this channel, induce spectacular hyperexcitability effects when injected in the central nervous system, including long-term potentiation [Bidard et al., 1987; Cherubini et al., 1987; for reviews see Dreyer (1990) and Moczydlowski et al. (1988)]. (ii) This K^+ channel, which is present in most brain regions, is probably involved in neurotransmitter secretion

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[1] Abbreviations: β-BTX, β-bungarotoxin; ChTx, charybdotoxin; DTX_I, dendrotoxin I; MCD peptide, mast cell degranulating peptide; RT–PCR, reverse transcription–polymerase chain reaction; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; $InsP_3$, inositol trisphosphate; PMA, phorbol myristate acetate.

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(Dreyer, 1990). (iii) This channel, unlike the Kv1.3 and ShH4 channels, has a very slow inactivation.

Results presented in this paper show that changes in the level of expression of Kv1.2 channel in *Xenopus* oocytes modify its biophysical properties of inactivation and also in a very drastic way its interaction with the different toxins.

EXPERIMENTAL PROCEDURES

Cloning of cDNA and Synthesis of cRNA. A full-length cDNA clone coding for Kv1.2 was obtained by RT–PCR (Kawasaki, 1990) on rat cardiac RNA. The cDNA sequence was identical to RBK2 (McKinnon, 1989) except for a change from Phe to Leu at position 412. A new yte expression vector, pBTG, was constructed by inserting the *Hin*dIII–*Pst*I cassette from SP64T (Melton et al., 1984) containing the 5′- and 3′-noncoding regions of the *Xenopus laevis* β-globin gene into pBluescript KS(1) (Stratagene). The Kv1.2 cDNA was inserted into the unique *Bgl*II site flanked by the two noncoding regions. For *Xenopus* oocyte injection, a single-stranded cRNA sense was synthesized using T3 RNA polymerase (RNA transcript kit from Stratagene) and the Kv1.2 cDNA clone was linearized by *Sma*I.

mRNA Injection in Xenopus Oocytes. X. laevis were purchased from C.R.B.M. (Montpellier, France). Pieces of the ovary were surgically removed and individual oocytes were dissected away in a saline solution (ND96) containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) at pH 7.4. Stage V and VI oocytes were treated for 2 h with collagenase (1 mg/mL, Sigma) in saline solution to discard follicular cells. RNA solutions (50 nL/oocyte) were injected using a pressure microinjector. Oocytes were then kept for 2–6 days in saline solution (ND96) supplemented with 100 IU/mL penicillin and 100 μg/mL streptomycin.

Electrophysiological Measurements in Xenopus Oocytes. In a 0.3-mL perfusion chamber, a single oocyte was impaled with two standard glass microelectrodes (0.5–2.0 MΩ resistance) filled with 3 M KCl and maintained under voltage clamp using a Dagan TEV 200 amplifier. Stimulation of the preparation, data acquisition, and analyses were performed using pClamp software (Axon Instruments; USA). Drugs were applied externally by addition to the superfusate (Gilson perstaltic pump; flow rate (0.3 mL/min). A saline solution (ND 96) was used in all procedures. Current to voltage (*I–V*) relationships were obtained by applying 200-ms depolarizing voltage steps every 10 from a holding potential of –60 mV to increasing depolarizing test potentials. The variability of the results was expressed as the standard error of the mean with *n* indicating the number of cells contributing to the mean. Student's *t* test was used for statistical analysis. Dose–effect relationships were fitted with the equation $y = ax/(b + x)$, where y was the percentage of Kv1.2 inhibition, x was the logarithm of toxin concentration, and b was the toxin concentration for 50% inhibition. When inhibition curves were only partial, a was fixed to 100% for fitting experimental data because of the very high toxin concentrations which would be necessary.

Binding Assays and Toxin Purification Methods. The different toxins from *Dendroaspis polylepis* venom were purified according to Schweitz et al. (1990). Their affinities to rat brain synaptosomes and RNA-injected oocytes were determined by competition experiments using ^{125}I-DTX$_I$ as previously described (Schweitz et al., 1990).

Nomenclature. The nomenclature for K^+ channel genes used through this paper is according to the one recently proposed by Chandy et al. (1991). In this nomenclature, Kv1.2 and Kv1.3 correspond to RBK2 (MacKinnon, 1989) and HLK3 (Attali et al., 1992).

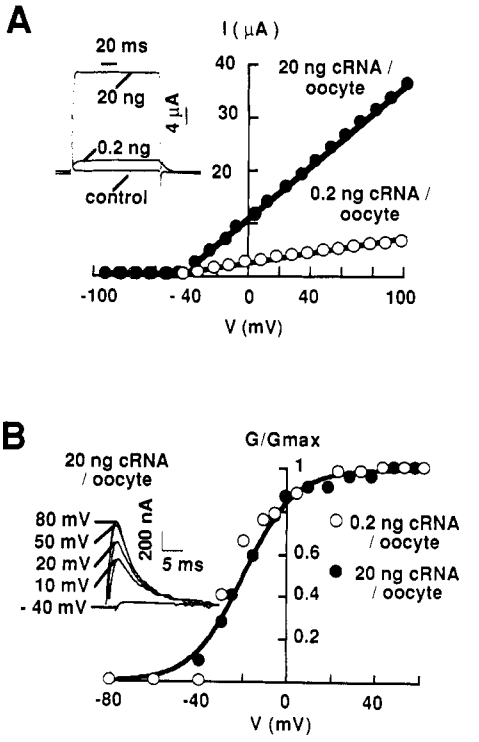

FIGURE 1: Functional expression of Kv1.2 current in *Xenopus* oocytes (A) *I–V* curves are illustrated for oocytes injected with 0.2 (○) and 20 (●) ng of cRNA. The holding potential was –60 mV, and depolarizing voltage steps were applied every 10 s by 10-mV increments. Inset shows current traces recorded in control and in oocytes injected with 0.2 and 20 ng of cRNA, respectively. (B) The conductance was calculated by measuring the amplitude of the tail currents recorded at –60 mV. The tail currents recorded at –60 mV after depolarizations to –40, 10, 20, 50, and 80 mV are illustrated in the inset.

RESULTS

Figure 1A presents *I–V* curves recorded in oocytes injected with either low or high concentrations of the cRNA encoding the Kv1.2 channel. Current amplitudes measured at +30 mV were 20 ± 5 μA (n = 8) and 5 ± 2 μA (n = 8) in oocytes injected with 20 and 0.2 ng of cRNA, respectively. The voltage threshold for activation was not significantly different at 20 ng of cRNA (–57 ± 2 mV) and at 0.2 ng of cRNA (–52 ± 3 mV). Measuring tail current amplitude (deactivation currents) at a fixed membrane (–60 mV), which is different from the value of the K^+ reversal potential, allows us to determine the relative K^+ conductance. The voltage dependence of the conductance was obtained from the initial amplitude of the tail currents and normalized to the peak tail current. Conductance curves, calculated from tail currents were identical in oocytes injected with both low and high cRNA concentrations (Figure 1B). Finally, the reversal potential assessed from tail current measurement was –83 ± 4 mV (n = 3) in the presence of 2 mM external K^+ with both low and high cRNA concentrations. Changing external K^+ concentration induced a shift of the reversal potential value as expected by the Nernst equation, demonstrating a K^+ selectivity (not shown).

Recording Kv1.2 current on longer time scales revealed a very slow relaxation process (Figure 2). This inactivation phenomenon, which occurs in the minute range, is clearly different from the fast inactivation of ShH4 channel (mil-

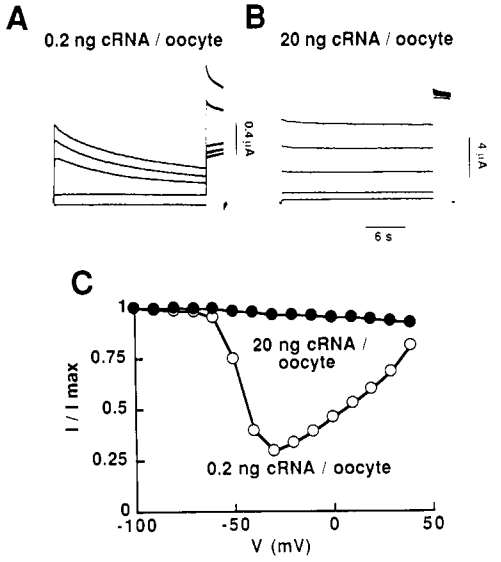

FIGURE 2: (A) Voltage-dependent inactivation of Kv1.2 in an oocyte injected with 0.2 ng of cRNA. The duration of the conditioning pulse was 25 s and the potentials were −100, −50, −30, −20, and −10 mV. The test pulse was 30 mV and the holding potential was −80 mV. (B) Voltage-dependent inactivation of Kv1.2 in an oocyte injected with 20 ng of cRNA. The duration of the conditioning pulse was 25 s and the potentials were −60, −50, −30, −10, and 10 mV. The test pulse was 30 mV and the holding potential was −80 mV. (C) Inactivation curves of Kv1.2 currents illustrated in (A) and (B).

lisecond range; Moran et al., 1992) or the slow inactivation typical of the Kv1.3 channel (second range; Honoré et al., 1992). This very slow inactivation was only observed with the low cRNA concentration (Figure 2). Inactivation curves were obtained using 25-s duration conditioning pulses (Figure 2C). With 0.2 ng of cRNA/oocyte, Kv1.2 was half-inactivated with a membrane potential of -44 ± 2 mV ($n = 5$). With 20 ng of cRNA/oocyte, no current relaxation occured during the 25-s duration predepolarizations (Figure 2B). Furthermore, no voltage-dependent inactivation was observed (Figure 2C).

Figure 3A shows the dose–response curves for Kv1.2 current inhibition by DTX_I in oocytes injected with low cRNA concentrations (0.2 ng/oocyte). Half-maximum inhibition was observed at 2 nM ($n = 10$).

The action of several other toxins from *D. polylepis* venom which belong to the DTX_I family and which bind to the DTX_I-sensitive K^+ channel (Schweitz et al., 1990) has also been analyzed. The most potent toxins are DTX_I itself and toxin H_4, and less potent is toxin J_I.

A good correlation was found between the binding affinity of the toxins, measured from their capacity to inhibit ^{125}I-DTX_I binding to rat brain membranes, and K^+ current inhibition in oocytes injected with 0.2 ng of cRNA. This result suggests that high-affinity ^{125}I-DTX binding sites in rat synaptosomal membranes titrate the Kv1.2 channel and is in agreement with the finding that this channel is the major DTX_I-sensitive component in the brain (Scott et al., 1990).

Figure 4A shows that while 10 nM DTX_I extensively inhibited the Kv1.2 current recorded in an oocyte injected with 0.2 ng of cRNA, the K^+ current had very little sensitivity to 10 nM DTX_I with an injection of 20 ng of cRNA/oocyte (Figure 4B). Figure 4C shows the different sensitivities to DTX_I for oocytes injected with increasing cRNA concentrations and indicates clearly that the higher the cRNA concentration, the lower the DTX_I sensitivity. Figure 4D shows the sigmoidal relationship between the mean maximal K^+ current amplitude obtained at different cRNA concentrations and the logarithm of the IC_{50} for DTX_I inhibition.

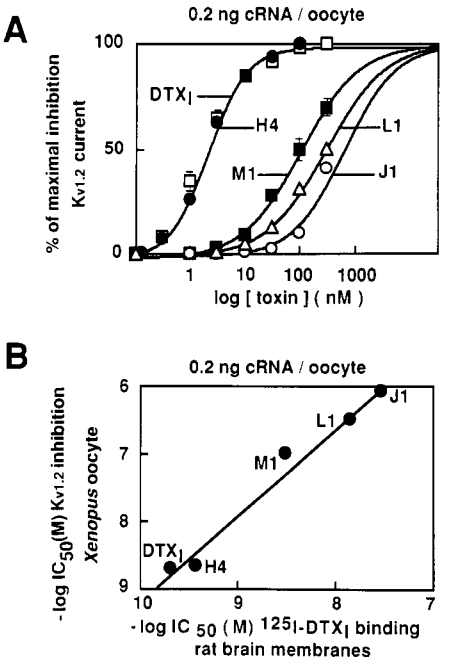

FIGURE 3: Kv1.2 current inhibition by dendrotoxins. (A) Concentration dependence for the inhibitory effect of dendrotoxins. Toxins are indicated on the curves. (B) Relationship between the logarithm of Kv1.2 inhibition IC_{50} and the binding affinity in rat brain membranes. In these experiments, the holding potential was −60 mV and the oocytes were depolarized to 30 mV.

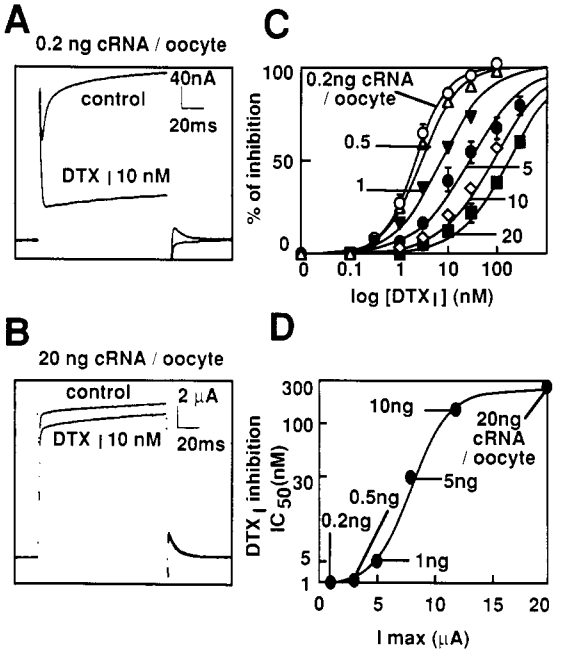

FIGURE 4: Two different DTX_I sensitivities in oocytes injected with 0.2 and 20 ng of cRNA. (A, B) Effects of DTX_I (10 nM) on K^+ currents expressed in oocytes injected with 0.2 ng and 20 ng of cRNA respectively. (C) Concentration dependence curves for DTX_I inhibition in oocytes injected with different cRNA concentrations. cRNA concentrations per oocyte are indicated. Numbers of oocytes used ranged from 4 to 8. (D) Relationship between the current amplitude and the logarithm of the IC_{50} for Kv1.2 current inhibition. The cRNA concentrations per oocyte are indicated. In these experiments, the holding potential was −60 mV and the oocytes were depolarized to +30 mV.

Kv1.2 K^+ currents are also known to be sensitive to MCD peptide and to ChTx (Stühmer et al., 1989). It has been shown that the channel is also blocked by β-BTX (Figure 5). The sensitivity of Kv1.2 to MCD peptide, β-BTX, and ChTx was also analyzed at low and high cRNA concentrations.

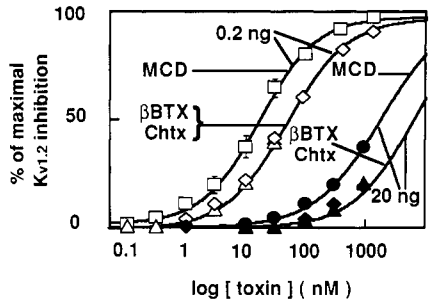

FIGURE 5: cRNA concentration directs the sensitivity of Kv1.2 channels to various neurotoxins. Inhibitory effects of β-BTX, ChTx, and MCD peptides in oocytes injected with 0.2 (open symbols) and 20 (closed symbols) ng of cRNA are shown. In these experiments, the holding potential was −60 mV and the oocyte depolarized to 30 mV.

Figure 6 shows that sensitivity of Kv1.2 to MCD peptide, ChTx, and β-BTX was drastically reduced at high cRNA concentrations.

Figure 6A shows that Kv1.2 current expressed in an oocyte injected with 20 ng of cRNA was inhibited by an intracellular injection of $InsP_3$. A similar result was obtained when injecting $CaCl_2$ (final concentration 1 μM, not shown). The histogram illustrated in Figure 6B shows that intracellular $InsP_3$ injections inhibited Kv1.2 currents with identical potencies in oocytes injected with low (0.2 ng of cRNA) or high cRNA concentrations (20 ng of cRNA). Kv1.2 current was also inhibited by treatment with the phorbol ester PMA (Figure 6C). The inhibition by PMA was independent of the amount of cRNA injected (Figure 6D).

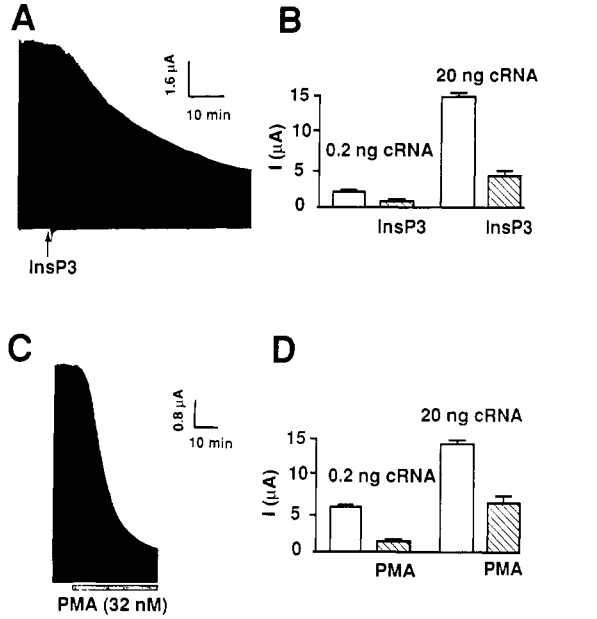

FIGURE 6: (A) Intracellular microinjection of $InsP_3$ (final concentration 1 μM) inhibited Kv1.2 current recorded in an oocyte injected with 20 ng of cRNA. (B) Histogram illustrating the inhibitions by intracellular injections of $InsP_3$ (final concentration 1 μM) of Kv1.2 currents expressed in oocytes injected with either 0.2 or 20 ng of cRNA (as indicated). (C) Effects of 32 nM PMA superfusion on Kv1.2 current recorded in an oocyte injected with 20 ng of cRNA. (D) Histogram illustrating the inhibition of Kv1.2 currents by 32 nM PMA in oocytes injected either with 0.2 or 20 ng of cRNA (as indicated). In these experiments, the holding potential was −80 mV and the oocytes were depolarized to 30 mV.

DISCUSSION

DTX_I, a snake venom toxin, and MCD peptide, a bee venom toxin, are potent blockers of voltage-sensitive K^+ channels. Both DTX_I and MCD peptide induce epileptiform activity and paroxystic seizures after intracerebroventricular injections to rats (Bidard et al., 1987, 1989). At lower concentrations MCD peptide induces hippocampal θ-rhythm (Bidard et al., 1987) associated with long-term potentiation (Cherubini et al., 1987). Both labeled DTX_I and MCD peptide binding sites have been localized by quantitative autoradiography (Mourre et al., 1988). They are both situated in synapse-rich areas. Sites for the two toxins are in mutual interaction since high-affinity binding of ^{125}I-MCD is abolished by DTX_I and vice versa. β-BTX and ChTx, which also block voltage-dependent K^+ channels [for review see Rehm (1992)], inhibit the binding of ^{125}I-DTX_I and ^{125}I-MCD (Schmidt et al., 1988; Schweitz et al., 1989a,b). MCD, DTX_I, β-BTX, and ChTx binding sites copurify during the biochemical isolation of voltage-dependent K^+ channels (Rehm & Lazdunski, 1988a,b).

Although three genes encode for α-subunits that produce K^+ channel activity which is inhibited by dendrotoxins (Stühmer et al., 1989), the major one which results from the purification of the DTX_I/MCD binding protein is Kv1.2 (Scott et al., 1990).

Since the biophysical properties of Kv1.3, the major K^+ channel in T-lymphocytes and also an important channel in the brain, are known to be altered when the levels of expression of the corresponding cRNA in oocytes are changed (changes in kinetics, changes in use dependence; Honoré et al., 1992), the first question was to know whether the biophysical properties of the DTX_I-sensitive K^+ channel Kv1.2 were also changed. In the Kv1.2 case however, the channel is a very slowly inactivating, typical delayed rectifier. We have observed that the intensity of the K^+ current was of course increased at high cRNA concentrations and that among the main biophysical properties of the channel only the voltage-dependent inactivation was changed in a detectable way. As in the case of ShH4 (Moran et al., 1992) and Kv1.3 channels (Honoré et al., 1992), the very slow inactivation of Kv1.2 channel was absent when high cRNA concentrations were injected into oocytes.

When expressed at low cRNA concentrations, Kv1.2 is very sensitive to DTX_I (IC_{50} = 2 nM). To better characterize this interaction at the DTX_I binding site under these cRNA concentrations, we have analyzed the properties of other analogs in the dendrotoxin family which had been previously purified from the venom of *D. polylepis* (Schweitz et al., 1990). They all have inhibitory properties for the Kv1.2 channel but their efficacy measured by their IC_{50} varies over about 2 orders of magnitude. An excellent correlation was found between the capacity of these toxins to block the Kv1.2 channel and their capacity to inhibit binding of ^{125}I-DTX_I to rat brain membranes.

Properties of interaction of the Kv1.2 channel with DTX_I are drastically altered when channel density is increased, i.e., when the K^+ current reaches maximal values higher than 10–20 μA. Then, the channel, which was largely blocked by 10 nM DTX_I in oocytes injected with low cRNA concentrations, became nearly completely resistant to the same toxin concentration. The IC_{50} for DTX_I inhibition of the Kv1.2 channel increased from 2 nM at 0.2 ng of cRNA to 200 nM at 20 ng of cRNA.

The Kv1.2 channel is also sensitive to other polypeptide toxins such as MCD, β-BTX, and ChTx, which have no structural homologies with DTX_I. While MCD peptide (IC_{50} = 20 nM), β-BTX (IC_{50} = 50 nM), and ChTx (IC_{50} = 50 nM) potently inhibited K^+ channel expressed from low cRNA concentrations, the channel became resistant to the same toxins

when it was expressed from high cRNA concentrations. Changes in IC_{50}s for all these toxins between low (0.2 ng/oocyte) and high (20 ng/oocyte) cRNA concentrations were at least by a factor of 100.

The Kv1.2 current was inhibited by increasing intracellular Ca^{2+} (released from $InsP_3$-sensitive pools or directly microinjected) and by activation of protein kinase C (with the phorbol ester PMA). These regulatory effects, unlike the biophysical and pharmacological properties, were not modified by increasing cRNA concentrations.

The first interesting conclusion from this work is that changes in properties of voltage-sensitive K^+ channels with changes of the level of expression of their mRNA are not limited to channels undergoing fast or slow inactivation (Honoré et al., 1992; Moran et al., 1992). These changes are also observed with a very slowly inactivating delayed rectifier. Two different plausible mechanisms could be proposed to explain both the changes in the voltage-dependent inactivation and in the pharmacological properties of Kv1.2 channels expressed at high concentrations of cRNA. One possible mechanism is that it is the clustering of tetrameric channels which is responsible for the observed changes of properties as previously proposed for fast- and slow-inactivating K^+ channels (Honoré et al., 1992; Moran et al., 1992). The second possible mechanism is that the channels are differentially processed or modified in their structure due to intracellular changes (e.g., depletion of endogenous factors) caused by the massive production of functional channels.

Drastic changes in interaction of Kv1.2 with DTX_I, MCD peptide, β-BTX, and ChTx at high cRNA concentrations suggest that the new structural organization directly or indirectly involves the structural part of the channel in charge of toxin recognition, which is believed to be associated with the pore region of the channel (Rehm, 1991).

The fact that the regulation of Kv1.2 channel by intracellular Ca^{2+} and protein kinase C activation is not modified by increasing cRNA concentration indicates that structural elements involved in the regulation are not involved in the rearrangements that lead to changes in the inactivation properties and in the inhibition with the toxins.

The Kv1.2 channel is a very representative and probably physiologically important K^+ channel in mammalian brain (Beckh & Pongs, 1990) and it would be very interesting to know whether properties described from expression in *Xenopus* oocytes are also observed in brain cells. It has been shown recently that levels of expression of different types of K^+ channels including Kv1.2 and Kv4.2 are modified in situations such as pharmacologically induced convulsions (Tsaur et al., 1992). The phenomenon described here could be the reason why, besides a high-affinity receptor for DTX_I and MCD, a whole range of lower or much lower affinity binding sites for these toxins has also been identified in different brain regions (Mourre et al., 1988). It has also been observed that certain brain regions are more sensitive to MCD than others (Mourre et al., 1988). Moreover, desensitization to toxin action observed at high levels of Kv1.2 channel expression could have important physiological implications if, as previously suggested, K^+ channel toxins have endogeneous equivalents in mammalian brain (Fosset et al., 1984; Cherubini et al., 1987).

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